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Characterization of Native and Modified Extensin Monomers and Oligomers by Electron Microscopy and Gel Filtration[1]

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ABSTRACT

We isolated hydroxyproline-rich extensin precursors from suspension-cultured tomato, cucumber, and sycamore-maple by salt-elution of intact cells and cell wall preparations. Cation exchange chromatography and HPLC gel filtration resolved these precursors into monomeric and oligomeric fractions, confirmed by amino acid analysis, immunological cross-reactivity, and TEM visualization. After rotary shadowing monomers appeared as flexuous rods with a contour length of 70 to 100 nanometers and a 'persistence length' (maximum linear displacement) of 44 to 51 nanometers. Oligomers were larger branched assemblies with occasional pores. Native extensin monomers gave uniform gel filtration retention times (Rts), but the Rts of HF-deglycosylated monomers varied depending on concentration, implying ionic interaction between the highly basic deglycosylated monomers and a weakly cationic gel matrix. Succinylation of the deglycosylated monomers reversed the net charge, and restored the retention time to that of glycosylated monomers, confirming the ionic interaction. Succinylation enhanced visualization of the deglycosylated monomers, which previously were barely discernible flexuous rods. The persistence length:contour length ratios of succinylated deglycosylated monomers (tomato sdP2) and glycosylated monomers (sP2) were the same, implying a similar molecular flexibility for both glycosylated and deglycosylated monomers at room temperature. These molecular properties are consistent with suggestions that extensin monomers reptate into the wall as a transmural protein 'weft' which becomes progressively cross-linked forming a network penetrated by the cellulose 'warp.'

Primary cell walls of plants contain about 1 to 10% protein of which extensin, a HRGP[2] is often a major part. The 'warp-weft' model proposes that insoluble extensin exists as a network of defined porosity penetrated by the cellulose microfibrils (17, 18), creating a composite (2) in which structural protein mechanically couples the load-bearing polymers.

Extensin, like other structural proteins such as collagen and elastin, is a generic term describing a family of related proteins, highly processed by co-translational hydroxylation, signal peptide cleavage, posttranslation glycosylation, secretion, and the formation of (unknown) intermolecular cross-links which produce the insoluble extensin network. We refer collectively to the soluble monomers and oligomers in brief, as precursors because they are (where studied) precursors to the insoluble network. However, we use the precise designation monomer and oligomer where appropriate.

Highly soluble precursors to the extensin network readily elute from intact cells (or isolated cell walls) washed with dilute salt solutions (26). Tryptic peptide mapping of the HF-deglycosylated extensin precursors from tomato (27) and genomic sequences from carrot (4), show highly periodic structures consistent with the polyproline II helix suggested earlier (16), confirmed by Van Holst and Varner (33), and corroborated by electron micrographs of extended flexuous rodlike molecules (28, 29, 33).

While these data are consistent with the warp-weft model mentioned above, direct visualization of the extensin network *in muro* is the ultimate test of the warp-weft model's validity. We also need to know the mechanism for the insertion of extensin precursors into the wall and how they become cross-linked.

As an approach to these problems, we used HPLC gel filtration and transmission electron microscopy of native and modified soluble extensin precursors from tomato, sycamore-maple, and cucumber.

Our TEM data confirm that extensin precursors to the network are mainly flexuous rods similar to those from carrot (28, 33). In addition we have, for the first time, visualized the deglycosylated monomers as thin, but discernible, extended rods. Furthermore, succinylation of the lysine epsilon amino groups of deglycosylated extensin precursors, enhanced the TEM image, confirming the persistence of rodlike character even in deglycosylated extensin precursors.

Succinylation also showed that deglycosylated extensin precursors gave anomalously high gel filtration retention, due to ionic interaction with the column, rather than decreased molecular size.

MATERIALS AND METHODS

Precursor Preparation and Purification. We isolated extensin precursors by salt elution of intact suspension-cultured cells as previously described (26), and separated the TCA-soluble fraction (after dialysis) on a BioRex-70 (BioRad) column (90 × 1.5 cm) eluted with a pH gradient (7.6–6.1) superimposed on a 0 to 1 M NaCl gradient. Sycamore-maple and tomato cell suspensions were grown on MET medium as previously described (27). Cucumber (cvs Comanche and Marketer) callus suspensions were grown on Murashige-Skoog medium (21) with 1 mg/L napthalene acetic acid and benzylaminopurine. Cucumber extensin precursor preparation consisted of cell wall isolation after a 90 s blend, water washing prior to salt elution, and further purification of salt eluates on a small (170 × 9 mm) BioRex column.

HF Deglycosylation. We deglycosylated tomato extensin precursors in anhydrous HF (containing 10% v/v anhydrous methanol) for 1 h at 0°C as described by Smith *et al.* (27).

Succinylation. We succinylated milligram quantities of tomato dP2 and P2 by dissolving the extensin precursor in about 2 ml

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[2] Abbreviations: HRGP, hydroxyproline-rich glycoprotein; P1, extensin precursor; P2, extensin precursor; dP1, deglycosylated P1; dP2, deglycosylated P2; sP2, succinylated P2′, sdP2, succinylated deglycosylated P2; TEM, transmission electron microscopy; Rt, retention time; HF, anhydrous hydrogen fluoride.

of water followed by the serial addition of 6 × 10 mg solid succinic anhydride, titrating to pH 7.5 with 1 N NaOH in a pH-stat and then exhaustively dialyzing the succinylated products (sdP2 and sP2) against distilled water.

Gel Filtration. We loaded 10 to 100 μl samples (1–20 mg/ml in [pH 7] 200 mM phosphate buffer) onto a Superose-6 (Pharmacia) column via a 200 μl sample loop of a Rheodyne 7125 injector. Flow rate was 300 μl/min at room temperature (23°C), monitored at 220 nm, and 1 ml fractions collected.

TEM Sample Preparation. We prepared the extensin precursors for TEM following the general methods of Tyler and Branton (32), *i.e.* samples (1–30 μg/ml) dissolved in 50% v/v aqueous glycerol were sprayed onto freshly cleaved mica chips with a modified airbrush. After drying the chips *in vacuo* on a rotary stage in a Balzers BAE 080 high vacuum evaporator, we shadowed the molecules at an angle of 5 to 6°, with Pt/C from a modified Balzers 052 twin-mantle electron-beam evaporator. After backing with carbon at 90°C we floated the replicas on distilled water, collected them on 300 mesh copper grids, and examined them in a JEOL 100 CX II transmission electron microscope operated at 100 kV.

Image Analysis. We used a Houston Instruments Hipad Digitizer and microcomputer to digitize high contrast micrograph prints (×250,000 calibrated with negatively stained catalase crystals).

A computer program summed the Euclidean distance between successively digitized points to yield the contour length of a given molecule. An approximation of the persistence length was given by the maximum Euclidean distance between the end coordinates of a molecule and any other point on that molecule. We took the persistence length:contour length ratio as a measure of relative molecular flexibility.

RESULTS

Gel Filtration. After purification via BioRex-70 cation exchange chromatography (Fig. 1A), HPLC gel filtration of the tomato precursors (Fig. 2) gave one major peak (Rt 43 min; Table I), confirmed as monomeric by TEM (see below). A minor preceding peak (Rt 35 min) had an extensin-like composition and was oligomeric (Fig. 2). Extensin precursors from other plant species gave distinctive BioRex profiles (Fig. 1, B and C) but similar HPLC profiles (Fig. 3).

Although the Rts of the native precursors were constant, the Rt of HF-deglycosylated tomato P2 (dP2) decreased with amount injected (Fig. 4), but was always greater than native P2 precursor Rt (Table I). However, after succinylating the HF-deglycosylated precursor, it (sdP2) co-eluted with the native monomeric precursor, at an Rt which no longer varied with column loading (Table I; Fig. 5). Interestingly, deglycosylated precursor (unlike the glycosylated precursors P2 and sP2) did not show a minor leading peak, but this oligomeric peak did resolve after succinylating dP2 (Fig. 5). Succinylation of the native P2 precursor did not alter its elution profile, although its retention time decreased slightly (Table I).

We calibrated the Superose-6 column with a range of globular protein standards (Fig. 6). Glycosylated extensin monomers co-eluted with thyroglobulin (669 kD) when co-injected, although when injected separately, the monomer Rt was between thyroglobulin and mouse IgM (900 kD). However, the oligomeric extensin peak co-eluted with mouse IgM.

Electron Microscopy. Rotary shadowed preparations of extensin precursors, previously purified on BioRex-70, appeared mainly as monomers mixed with smaller amounts of oligomers in TEM (Fig. 7). Further fractionation on Superose-6 separated monomers from oligomers (Fig. 8); TEM showed an oligomeric linkage pattern, mainly branched but with occasional closed pores, and terminally linked regions with contour lengths greater than 100 nm (Fig. 8).

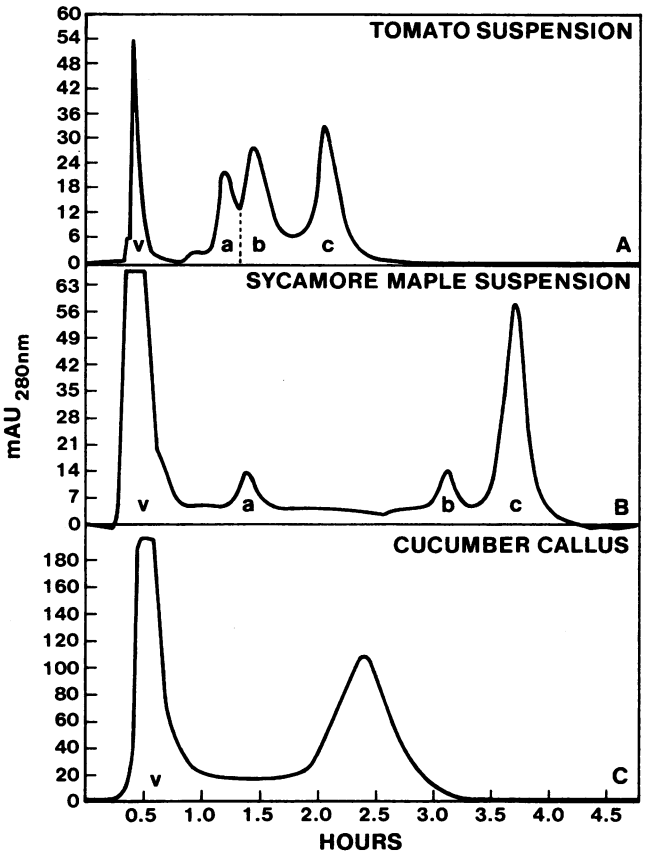

FIG. 1. BioRex-70 cation exchange chromatographic separation of soluble extensin precursors: A, tomato P1a, P1b, and P2 are peaks a, b, and c, respectively; B, sycamore maple (peak c); C, cucumber callus. Void peaks (v) did not contain extensin.

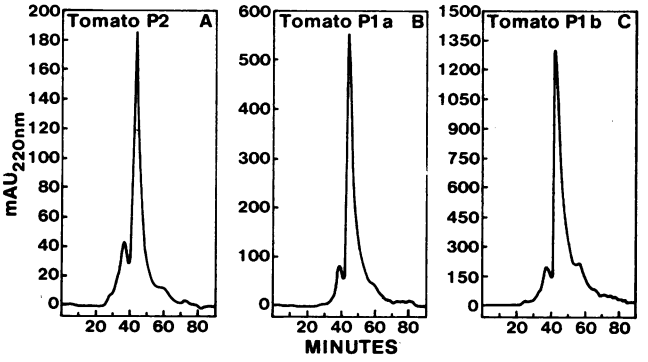

FIG. 2. Superose-6 gel filtration of BioRex-purified extensin: A, tomato P1a; B, tomato P1b; C, tomato P2.

The precise molecular morphology of the monomers depended on droplet geography which showed two distinct areas: an inner region left as the glycerol evaporated, and an outer surrounding region, transiently occupied by a droplet only during initial impact (see "Discussion"). Molecules in the outer region were generally more linear than those in the central region (Fig. 9). Therefore we measured contour lengths and apparent 'persistence length' of molecules from the inner droplet region as it probably gives a truer indication of molecular flexibility.

Although flexuous and bent apparently at random, the mon-

Table I. *Superose-6 Gel Filtration Retention Times for Soluble Extensin Precursors*

Source and Type	Retention Times	
	Main peak	Leading Peak
	min	
Tomato suspension:		
(P2)	43	35
(P1a)	42	37
(P1b)	44	36
Cumber callus suspension	43	36
Sycamore callus suspension	42 (45)[a]	35
Deglycosylated P2:		
(100 μg)	61	
(500 μg)	58	
(1000 μg)	50	
Succinylated P2	37	31
Succinylated dP2	42	36

[a] Sycamore maple main peak had a shoulder that eluted at 45 min.

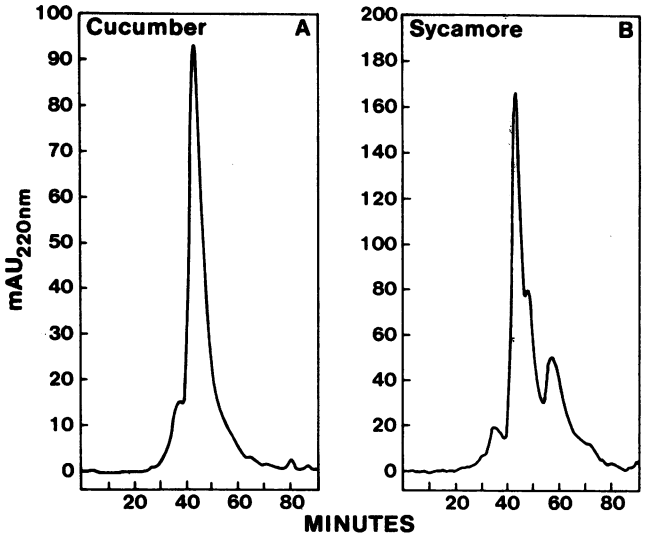

FIG. 3. Superose-6 gel filtration of BioRex-purified extensin: A, from cucumber callus; B, from sycamore-maple cell suspension cultures.

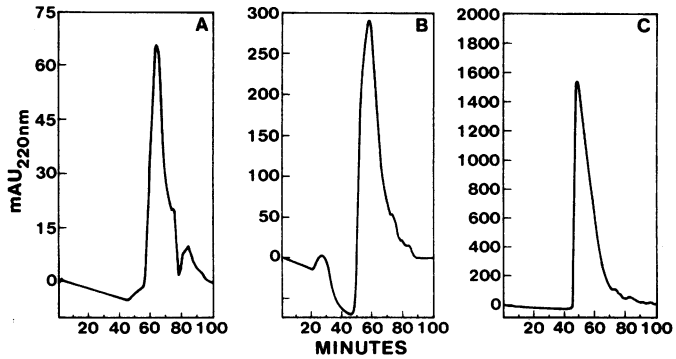

FIG. 4. Superose-6 gel-filtration of HF-deglycosylated P2 (dP2) at (A) 100 μg, (B) 500 μg, and (C) 1000 μg column loading.

omers were sufficiently rigid to prevent intramolecular loop formation. An average contour length of 80 to 100 nm (Fig. 7) and an apparent 'persistence length' (maximum linear displacement) of 44 to 51 nm quantifies the limited flexibility of these molecules

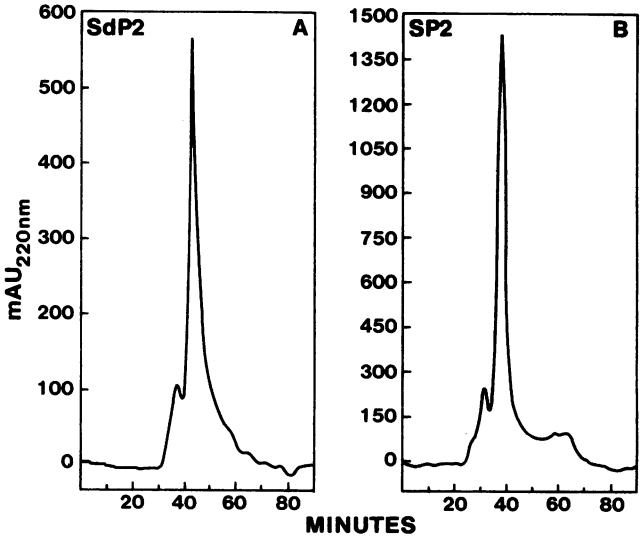

FIG. 5. Superose-6 gel-filtration of (A) succinylated, HF-deglycosylated P2 (sdP2) and (B) succinylated native P2 (sP2).

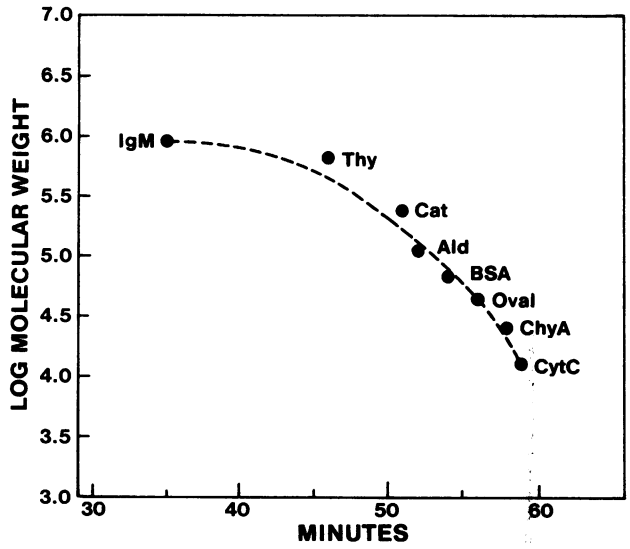

FIG. 6. Superose-6 gel filtration of 8 globular protein standards: IgM, mouse IgM; Thy, thyroglobulin; Cat, catalase; Ald, aldolase; Oval, ovalbumin; ChyA, chymotrypsin.

from three different species (Table II). Because previous work (see "Discussion") ascribes a structural stabilizing role for the oligoarabinosides we were interested in the effect of their removal on molecular shape and flexibility. HF-deglycosylated monomeric tomato P2 (dP2) molecules, although close to the limit of TEM resolution, were nevertheless still rodlike (Fig. 10, A and B); succinylation of dP2 significantly enhanced TEM resolvability (Fig. 10C) both of monomers and oligomers. Succinylation also enhanced the resolvability of native P2, but had no other obvious effect on molecule morphology (Fig. 10D).

DISCUSSION

The gel filtration characteristics of extensin precursors described here, and also by Stafstrom and Staehelin (29), are apparently anomalous when compared with globular protein standards where Stokes radius, which assumes perfect spheres and no ionic interactions, is an adequate model of the hydrodynamic properties (9). On the other hand, due to molecular entangle-

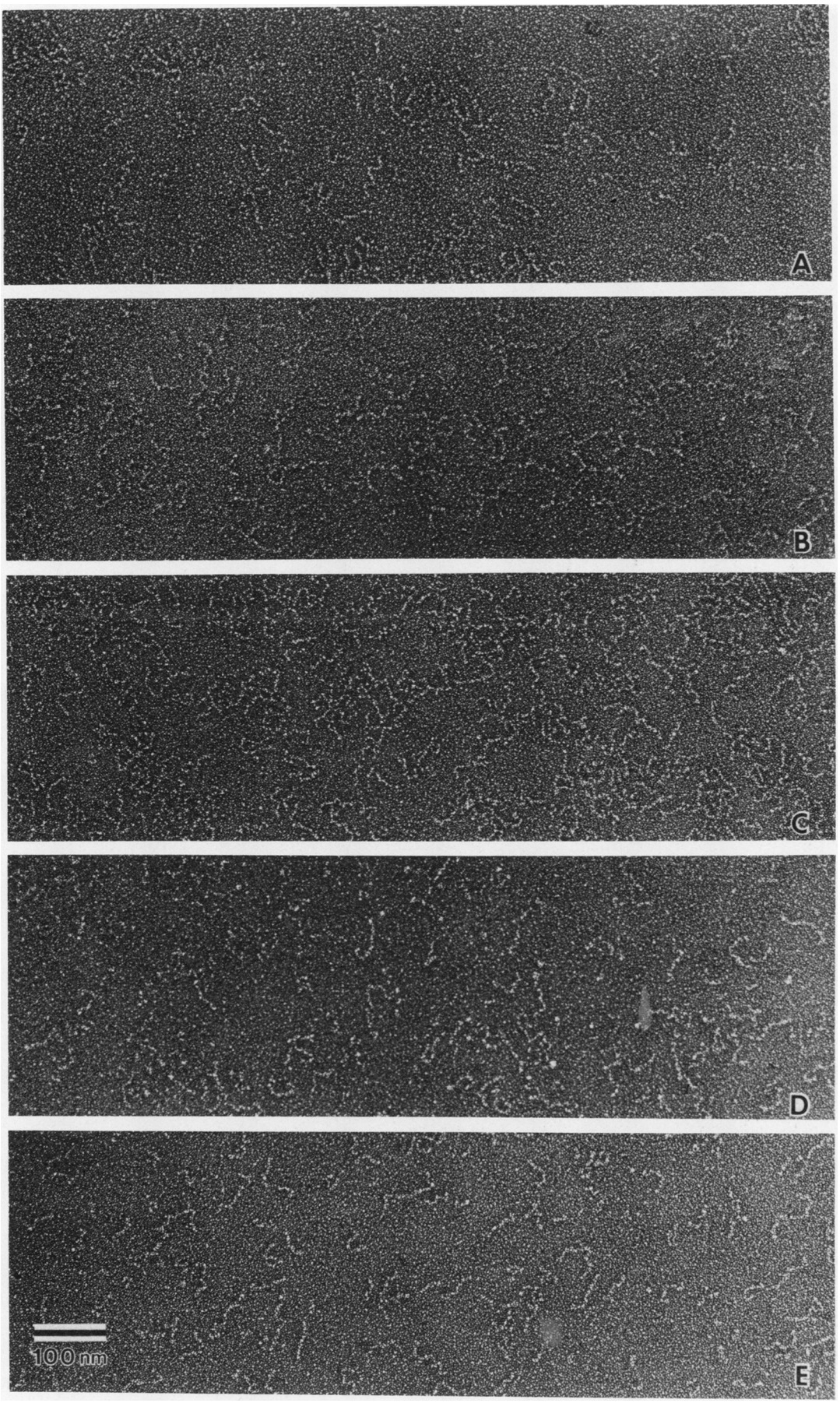

FIG. 7. Transmission electron micrographs of BioRex-purified extensin precursors from (A–C) tomato P2, P1a, P1b; D, cucumber callus suspension; E, sycamore cell suspensions.

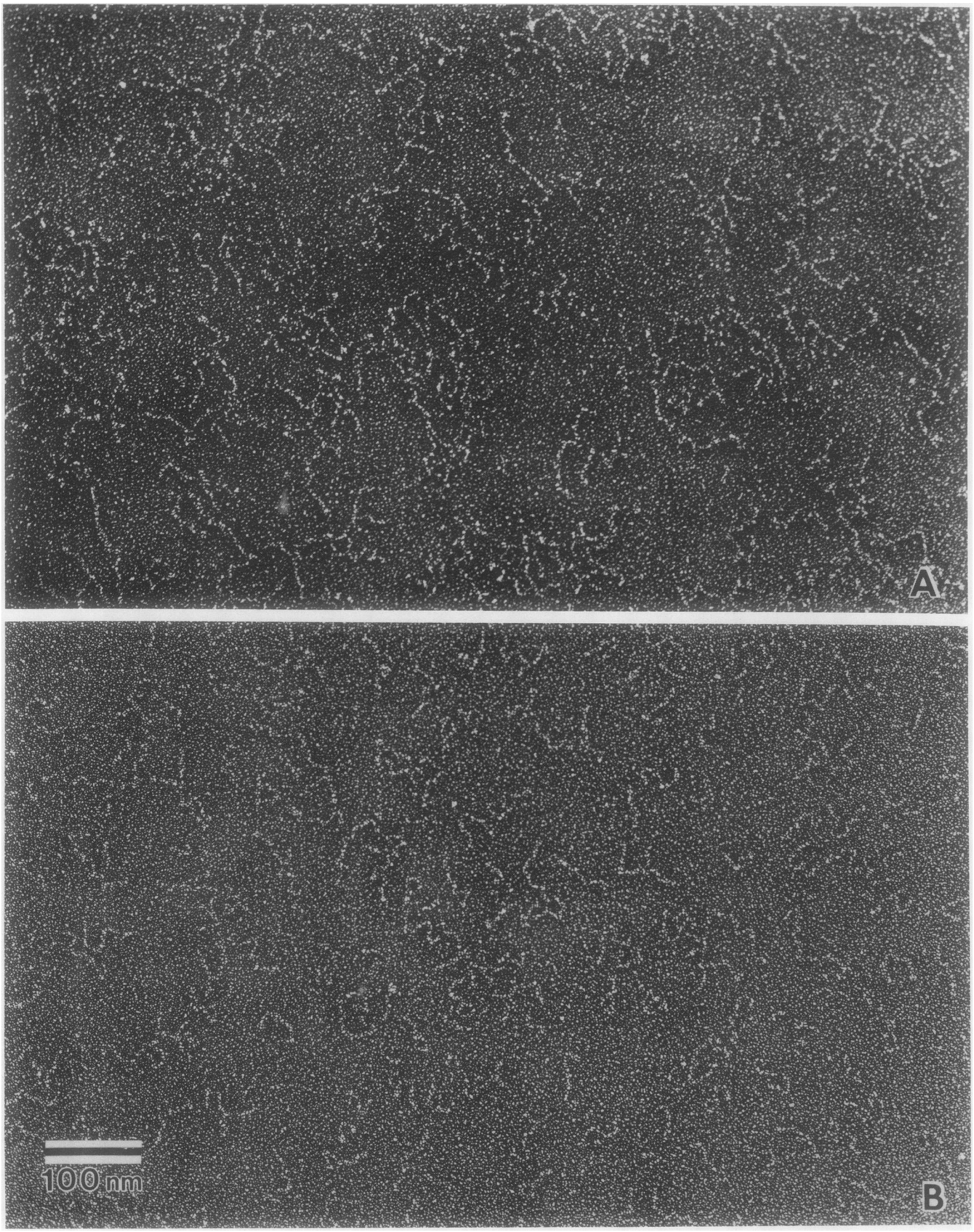

FIG. 8. Transmission electron micrographs of Superose-6 separated tomato P2 extensin (A) oligomeric fraction and (B) monomeric fraction.

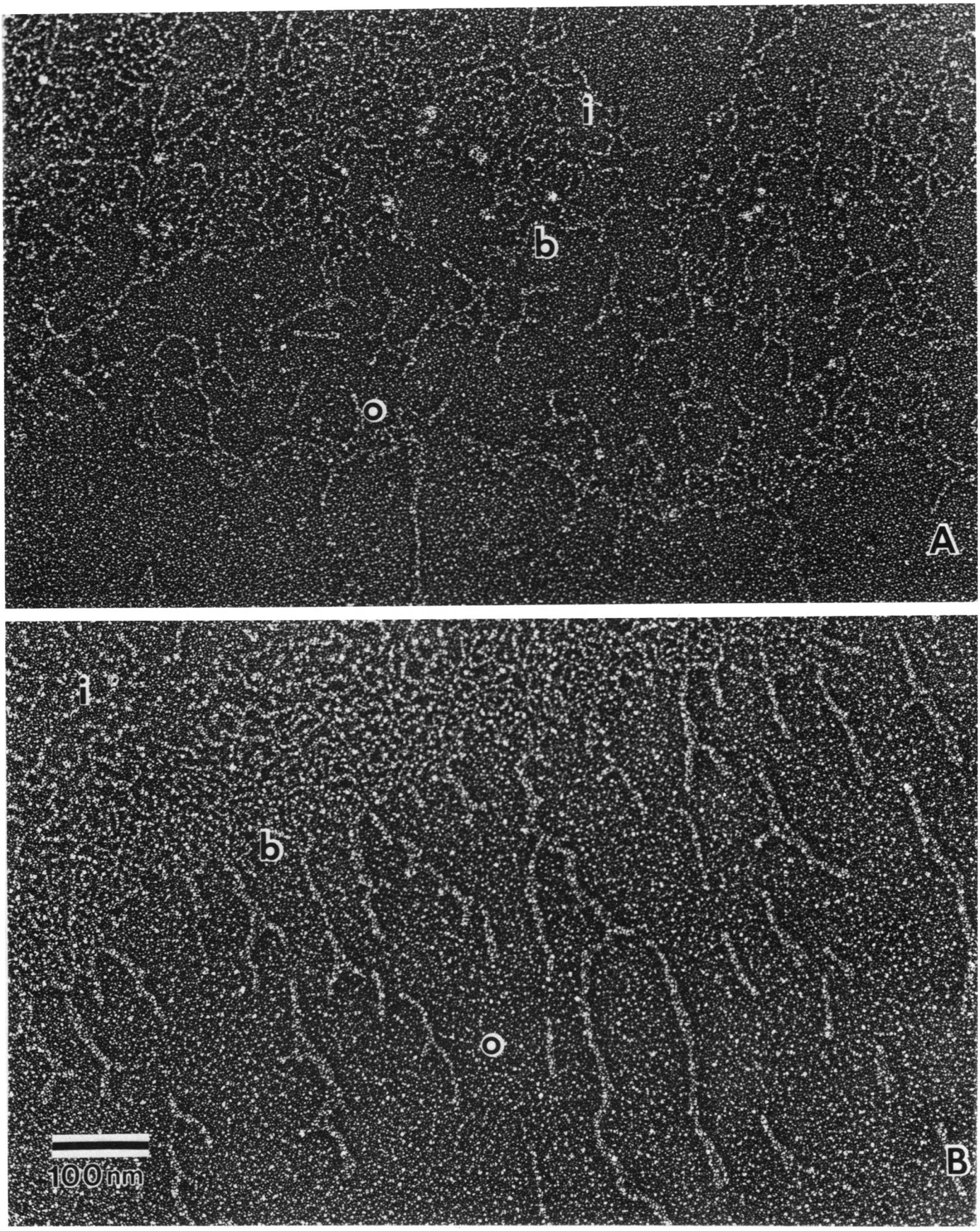

FIG. 9. A, Transmission electron micrographs of 'droplet effects' on precursor molecular morphology. 'b' Marks the boundary of a spray droplet of tomato P2 on mica, 'i' marks typical P2 molecules of the droplet inner region, and 'o' marks typical P2 molecules of the droplet outer region. B, Transmission electron micrograph of succinylated native P2 at the droplet edge. Droplet regions i, b, and o are the same as in A.

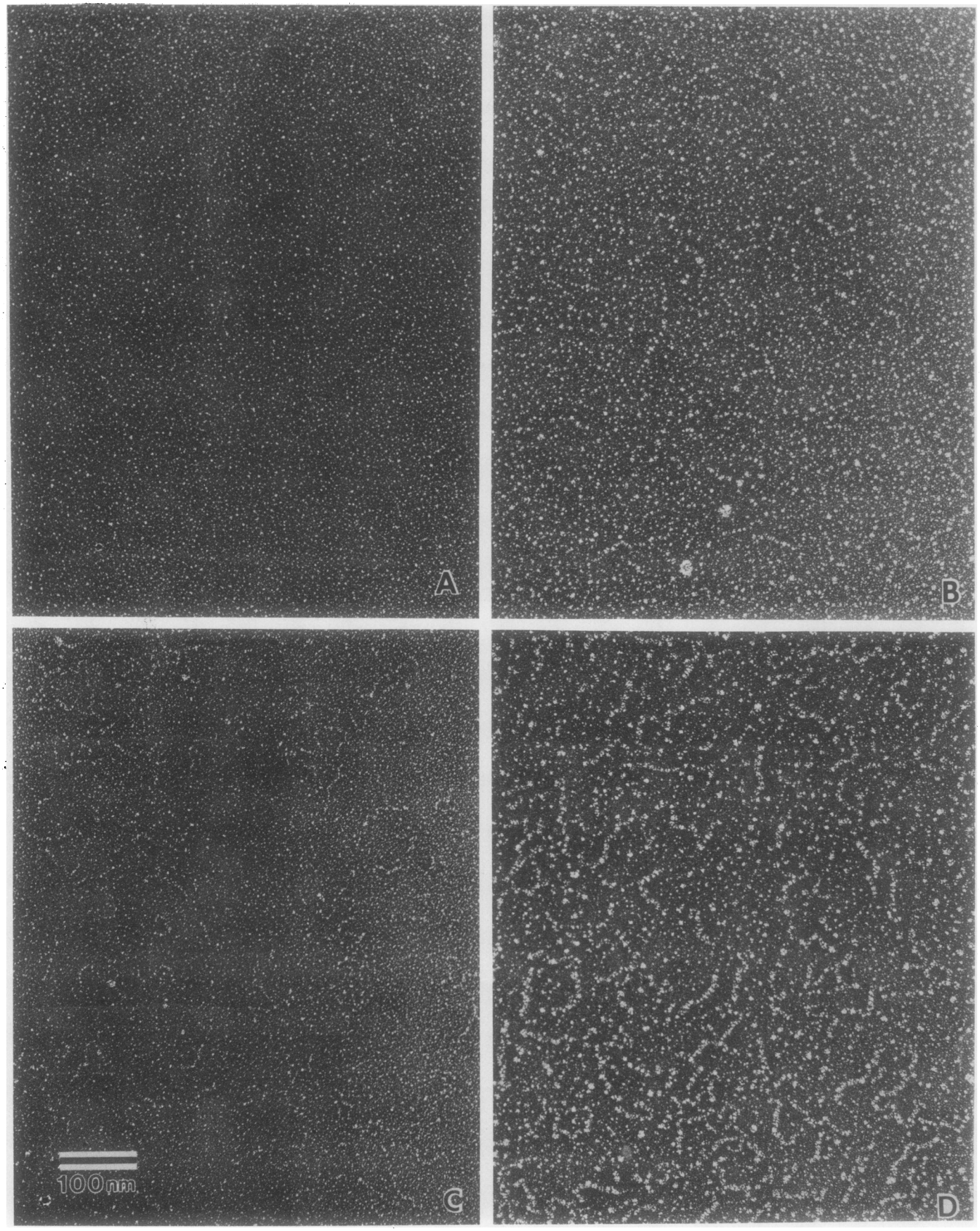

FIG. 10. Transmission electron micrographs of (A) glycerol blank, (B) dP2, (C) sdP2, and (D) sP2.

Table II. *Mean Contour (CL) and Persistence Lengths (PL) of Native and Modified Extensin Monomers Determined by TEM*

Source and Type	No. Measured	CL	PL	PL/CL
		nm		*ratio*
Tomato P2	149	85	51	0.60
Tomato P1a	153	79	49	0.62
Tomato P1b	133	74	44	0.59
Sycamore maple	145	71	49	0.69
Cucumber	99	77	49	0.63
Deglycosylated tomato P2	ND[a]	ND	ND	ND
Succinylated tomato P2	116	86	56	0.66
Succinylated deglycosylated tomato P2	122	69	46	0.67

[a] Not determined.

ment, rigid rodlike molecules or flexible rodlike molecules composed of rigid segments, like extensin monomers (14), do not tumble to form hydrodynamic spheres defining the Stokes radius, but tend to reptate (19). Reptation, defined as end on migration of a linear polymer, allows endwise insertion of linear macromolecules into porous gel matrices (13), resulting in a retardation different from that predicted by the mass (kD) of the macromolecule. Thus, reptation accounts for the gel filtration characteristics of collagen (23) and the electrophoretic mobility of large DNA in agarose gels (30). More quantitatively useful hydrodynamic models for complicated asymmetric biological molecules is the goal of work in progress (34); the misapplication of Stokes radius fo such molecules is surprisingly common (3, 15, 29).

Our gel filtration data showing a virtual co-elution of monomeric extensin precursors with thyroglobulin, are not consistent with a Stokes radius interpretation; thyroglobulin has a Stokes radius of 8.5 nm and 669 kD mass (24), while the extensin persistence length/2 is 25 nm (Table II) for an estimated 90 kD molecule (27). However, our gel filtration data are consistent with endwise insertion of extensin rods into the gel matrix, as their elution position on the column should depend more on properties that influence reptation (*e.g.* segmental flexibility) than molecular mass *per se*. On that basis, completely deglycosylated extensin precursors (which have lost two-thirds of their mass) should gel filter like the native monomers, provided the molecular flexibilities were comparable. But our gel filtration data (Table I) show marked differences between the Rts of native and deglycosylated extensin monomers implying differences in molecular flexibility and conformation. On the other hand our TEM data (Table II; Fig. 10) show that glycosylated and deglycosylated precursors have a similar molecular flexibility. Two experiments resolved this apparent discrepancy: First, the Rts of deglycosylated extensin (Table I) were dependent on column loading (Rt decreased as load increased), implying an ionic interaction of the highly basic protein with the column matrix, even in 0.2 M buffer (9); presumably deglycosylation allows closer contacts, and hence stronger coulombic interactions, between the column matrix and the carbohydrate-free polypeptide. Second, reversal of the molecular charge by succinylating all the amino groups of extensin, restored the Rts of the deglycosylated monomers to that of the native (glycosylated) molecule (Table I); these data confirm the ionic interaction and also demonstrate that mass *per se* does not determine the retention time, as complete succinylation adds only about 3 kD (calculated from the P2 composition [26, 27]). We conclude that gel filtration Rts of extensins depend more on the molecular properties that affect reptation than molecular mass *per se*. Because we could visualize deglycosylated extensin monomers as rods (Fig. 10) (*cf.* 29), we also infer that glycosylation of the hydroxyproline residues is not an absolute requirement for the formation of rods. However, arabinosylation may contribute to the stability of the polyproline II helix (16, 17, 29, 33).

The apparent loss of extensin oligomers after gel filtration of dP2 is also probably an ionic effect, as they reappear after succinylation (Figs. 4 and 5A). The persistence of oligomers after deglycosylation also confirms that the intermolecular cross-links are HF-stable and therefore do not directly involve the oligoarabinosyl side chains.

While gel filtration provides a quick assay for monomeric and oligomeric extensins, TEM allows a more precise determination of size, shape, and flexibility of biopolymers (31). Although negative staining was not effective in our hands, the 50% glycerol-spray/low angle (Pt/C) shadowing technique did visualize extensins well enough to measure contour length, 'persistence length,' and also to test for nonrandom bends or kinks. These measurements assume that, after passage from three dimensions in solution to a two-dimensional surface, the adsorbed molecules represent their original rigidity or flexibility minus one degree of freedom (10).

Aerosol sprays of extensin in 50% glycerol produced droplets (25–150 μm diameter), which after drying *in vacuo* yielded two distinct zones, inner and outer, characterized by a slightly different molecular concentration and morphology. Extensin molecules in the inner region were somewhat more flexuous than those of the outer region (Fig. 9A), the extreme example being succinylated P2 molecules which were radially aligned in the outer region (Fig. 9B), presumably by a flow-induced polarization due to a surface tension driven elastic contraction (7) of the droplet after it 'pancakes' onto the mica surface. The shape of highly positive charged molecules in the outer droplet region probably reflects their approximate landing position 'frozen' by the negatively charged mica surface. However, negatively charged succinylated molecules (sP2) should not interact strongly with the negative mica surface, and this probably allowed flow polarization and radial alignment (Fig. 9).

Interestingly, the analogous (and perhaps homologous) fibrous hydroxyproline-rich cell wall glycoproteins of *Chlamydomonas* are specifically oriented after adsorption on mica flakes (11). However, unlike extensin, the *Chlamydomonas* molecules show definite (nonrandom) kink sites and terminal knobs (11), but no isodityrosine (25). Tomato P2 extensin has random kinks and also very short intramolecular links created by isodityrosine: ½ IDT-LYS-½ IDT. While it would be natural to equate kinks with IDT content, our observations suggest otherwise: CPK molecular models (6), showed that IDT locks the conformation at the intramolecular cross-link to create a linear segment rather than a segmented IDT-induced kink (28). P1 contains no IDT

yet has kinks, while P2 contains IDT and kinks, but these kinks often disappear in succinylated molecules subjected to flow polarization (Fig. 9). Ergo, IDT and kinks are not correlated.

Gel filtration and TEM reveal molecular properties which may help explain how monomeric extensin precursors enter the wall, and how they assemble to form a three-dimensional network. Because monomeric extensin precursors (and to a small extent the lower oligomers) elute rapidly (50% in 10 s) from the cell wall even of intact cells, we suggested (17) their radial orientation in a microporous wall. A limiting pore diameter of 4 to 6 nm (determined by the penetration of colloidal gold [8], dextrans and polyethylene glycols [1], combined with a limited molecular flexibility [Table II]) implies direct insertion of monomers *into* the wall (*i.e.* intussusception rather than apposition), and hence a nonrandom orientation. The elution even of extensin oligomers from intact cells also argues for a preferential orientation of extensin, most likely as a transmural protein.

Both secretion and coulombic interactions with pectin are the most likely driving forces for the insertion of extensin monomers, which, being highly basic, bind strongly to pectic carboxyl groups (26). However, pectin is usually secreted in a highly methyl esterified form which 'ages' by progressive demethylesterification (20, 22), and may therefore produce an electrostatic gradient (5) across the wall which could both orient extensin precursors and help them migrate.

Finally, we note that extensin monomers are of similar size and form regardless of their origin from diverse species grown under different conditions. This similarity is consistent with the idea of extensin as a transmural protein (17), also recently suggested for *Chlamydomonas* (12). Because of its precise, invariant length, such a transmural protein could be a determinant of primary cell wall thickness.

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